The Plant Cell, Vol. 16, S154–S169, Supplement 2004, www.plantcell.org © 2004 American Society of Plant Biologists

Interactions of Mitochondrial and Nuclear Genes That Affect Male Gametophyte Development

Maureen R. Hanson[1] and Stéphane Bentolila

Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853-0001

INTRODUCTION

Apart from their agronomic importance in hybrid seed production, mutations that encode cytoplasmic male sterility (CMS) provide a means to probe the role of the mitochondrion in reproductive development. Fertility restorers are examples of nuclear genes that affect cytoplasmic gene expression, and their identification can illuminate the interactions between the two genomes. In this review, we consider what is known about the nature of mutant mitochondrial loci that disrupt pollen development, focusing on their creation through recombination events that have often involved ATP synthase subunit genes. We also discuss how the mutant mitochondrial genes' function or expression is affected by the presence of nuclear fertility restorers and the information gained about these nuclear genes through recent map-based cloning efforts. We also describe the evidence that mitochondrial gene expression can affect the function of nuclear gene products that control floral development.

In natural plant populations, the most widespread manifestation of disturbed mitochondrial–nuclear interaction is altered floral development, particularly the loss of the male gametophyte. A substantial fraction of species exhibit populations that include both hermaphroditic (male-fertile) and functional female (male-sterile) individuals, a situation termed gynodioecy by Darwin (discussed by Budar and Pelletier, 2001; Charlesworth, 2002). Although the genetics of the male sterility has not yet been determined in many known gynodioecious species (reviewed by Kaul, 1988), a typical finding is cytoplasmic inheritance of male sterility and restoration of fertility by one or more nuclear alleles. Although non-Mendelian traits in plants could be encoded by either cytoplasmic genome, in all cases to date in which such a sterility-encoding gene has been identified in an organelle, the mitochondrion was the location of the mutation that disrupts pollen development.

CMS forces outcrossing; however, the potential advantage of outcrossing does not appear to explain completely the success of the gynodioecious reproductive strategy. Quite possibly, the selective advantages and disadvantages of a mixture of female and hermaphroditic plants may vary between individual genera (Kaul, 1988; Budar et al., 2003). For some species, there is sound evidence that female plants produce more seeds, perhaps because of reduced energetic investment in male floral organs. Indeed, in many species, CMS halts pollen development at a very early developmental stage, potentially saving considerable output of resources. However, no increase in seed set in females has been detected in some species. Furthermore, in other species, the disruption occurs late in development, after considerable energy has been expended. Such a finding raises the question of whether cytoplasmic genes that confer male sterility may somehow confer a survival advantage to individuals, so that more CMS plants reproduce, even if individuals do not produce more seeds. The potential adaptive value of gynodioecy and its impact on the population structure and evolution of species remain topics for further inquiry (recently reviewed by Charlesworth, 2002; Budar et al., 2003; Saur Jacobs and Wade, 2003). Understanding the nature of CMS-encoding cytoplasmic genomes and the nuclear genes that suppress male sterility may be instructive in creating models for the maintenance of the balance of female and hermaphroditic plants in natural plant populations.

In addition to the naturally occurring CMS that has been observed in wild plant populations, the trait has been synthesized as a result of crosses in which the nuclear genome of one species has been moved into the cytoplasmic background of another. The failure of pollen development that arises as a result of interspecific nuclear/cytoplasmic combinations is termed alloplasmic male sterility. Quite likely, the finding of CMS genotypes after interspecific crosses results from the exposure of aberrant cytoplasmic genes whose expression is suppressed by nuclear genes present in the original species. However, there also is evidence that the process of hybridization itself can sometimes be mutagenic, perhaps creating new cytoplasmic genome configurations as a result of disturbances in cytoplasmic genome replication and organization (reviewed by Hanson and Conde, 1985). CMS individuals that lack seeds and fruit also have arisen after deliberate mutagenesis. Male-sterile individuals often are obvious among large plant populations in agricultural fields and thus are detected readily.

The phenomena of CMS and fertility restoration have been exploited by plant breeders to synthesize hybrid lines of a number of crop species (Frankel and Galun, 1977; Mackenzie, 2004). Any seed collected from a male-sterile plant must result from cross-pollination. For species in which the desired product is a vegetative organ or a flower, the absence of self-pollination does not reduce the yield or value of the hybrid crop, and the absence of pollen may even be desirable. If fruit or seed is the valuable product from a hybrid crop, however, the presence of a nuclear fertility restorer is essential to confer self-pollination on the hybrid plants. Depending on the historical custom for

[1] To whom correspondence should be addressed. E-mail: mrh5@cornell.edu; fax 607-255-6249.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.015966.

particular species, the restoring alleles of these genes are referred to as *Rf* (*Restorer of fertility*) or *Fr* (*Fertility restorer*). The discovery of new CMS-encoding genotypes and corresponding fertility restorers remains a goal in plant breeding. Furthermore, molecular methods to facilitate the movement of fertility-restoring genes into breeding lines can reduce the time to synthesize new parental lines for hybrid production.

STRATEGIES FOR IDENTIFYING CMS-ASSOCIATED MITOCHONDRIAL DNA

To identify the DNA sequences that encode CMS, an obvious strategy is to compare cytoplasmic genomes in fertile and CMS lines. Unfortunately, with a few exceptions, the comparative genomics strategy has not been successful. Differences between two cytoplasms often may reflect evolutionary divergence and have no correlation with CMS. What is needed, therefore, is to compare recently diverged CMS and fertile lines. Ideal genetic materials are lines of fertile revertants that have arisen recently from known CMS genotypes. As described below, such revertants were key to identifying the CMS-associated regions of maize CMS-T (Texas), maize CMS-S (U.S. Department of Agriculture), and common bean. This strategy is limited to particular species; in others, spontaneous or induced reversion events have never been detected.

A second strategy for locating CMS-associated mitochondrial DNA (mtDNA) regions is to observe the segregation of a particular DNA sequence with the phenotype. In most species, chloroplast and mitochondrial genomes are uniparentally inherited, and usually both are inherited through the same parent. Protoplast fusion has allowed a means to break the coinheritance of chloroplast DNA and mtDNA and to obtain recombinant mitochondrial genomes (reviewed by Hanson, 1984). Analysis of somatic hybrids between CMS and fertile protoplast parents has shown that fertility does not segregate with the chloroplast DNA. In *Petunia* and *Brassica*, lines that contain recombinant mitochondrial genomes have been used to identify molecular markers for CMS, leading to the identification of CMS-associated loci (Hanson, 1991; Budar and Pelletier, 2001). The recombinational genetics strategy is limited to those species in which protoplast fusion and plant regeneration are feasible technically, and only if recombinant mitochondrial genomes are found in somatic hybrids. Loci so identified can be tested further by analysis of restorer gene effects on the expression of genes that segregate with CMS.

A third strategy, in which proteins synthesized by mitochondria in CMS and fertile lines are compared, may detect a CMS-associated protein if it is synthesized in sufficient levels in organello to be detectable. For example, Forde et al. (1978) were able to find a CMS-associated protein by this strategy. With the development of high-throughput proteomics methods, it may be possible to identify CMS-associated proteins in the future by comparing the entire mitochondrial proteome in CMS and fertile plants.

At present, the most general strategy is to compare the mitochondrial genes, transcript profiles, or genomes in fertile and CMS lines to search for unusual recombinant genes, followed by assessment of the effect of nuclear fertility restorers on expression of the abnormal genes. This method is not foolproof, because a restorer locus may affect the transcript profile of not only a CMS-associated gene but also that of a normal gene. Thus, a gene could be wrongly implicated in CMS if it happens to be affected by a restorer allele. The strongest evidence comes from a combination of altered expression in restored lines along with either cosegregation with CMS in mtDNA recombinant lines or correlation of reversion to fertility with specific mtDNA alterations.

A common theme that emerges when known CMS-encoding genes are examined is their origin from recombination between ''legitimate'' mitochondrial genes and unknown open reading frames (*orfs*) or between different genuine mitochondrial coding regions. Because of the propensity for recombination in plant mitochondrial genomes, the mere identification of a gene that has been modified by recombination is not adequate to conclude that it is a causal factor in CMS. Perfectly fertile lines can carry apparently abnormal genes that have arisen from recombination (for examples, see Hanson and Folkerts, 1992; Marienfeld et al., 1997). Some may not be expressed, and the expression of others may have no consequence. For example, if an extra ORF is added to the 5′ or 3′ end of a gene whose product is processed N terminally or C terminally, the mature protein will be identical to the expected wild-type protein. A number of articles have appeared in which an abnormal gene or mtDNA arrangement has been detected in a collection of CMS lines but not in a collection of fertile lines. Without some corroboration, these differences cannot be concluded to be causal with respect to CMS, because they may merely reflect the evolutionary divergence of different mitochondrial genomes. Indeed, the literature includes a number of articles claiming a link between a particular apparently abnormal gene and CMS, correlations now known to be coincidental. Mitochondrial loci for which there is strong evidence for a role in CMS are discussed below.

COMPLEX MITOCHONDRIAL LOCI ASSOCIATED WITH CMS

The Multiply Recombined Chimeric Genes in Maize CMS-T and Petunia

Mitochondrial loci correlated with CMS were first identified in maize carrying the CMS-T male-sterile cytoplasm. A differential hybridization approach was used to detect a gene expressed in CMS lines but not in fertile lines. When the clone was sequenced, an *orf* (termed *urf13*) was detected that predicted a 13-kD protein (Dewey et al., 1986) that was detected originally as a 15-kD CMS-specific protein by in organello protein synthesis (Forde et al., 1978). The coding region of the gene is composed primarily of noncoding sequences normally found 3′ to the 23S rRNA (Figure 1). The maize *urf13* gene is located upstream of a conserved mitochondrial gene termed *orf221*, which encodes a membrane-bound protein (Prioli et al., 1993), now identified as ATP4 (Heazlewood et al., 2003). Critical confirmation that *urf13* causes pollen disruption came from characterizing mitochondrial genomes in fertile revertants that arose from cell culture (Gengenbach et al., 1981), in which were detected deletions that

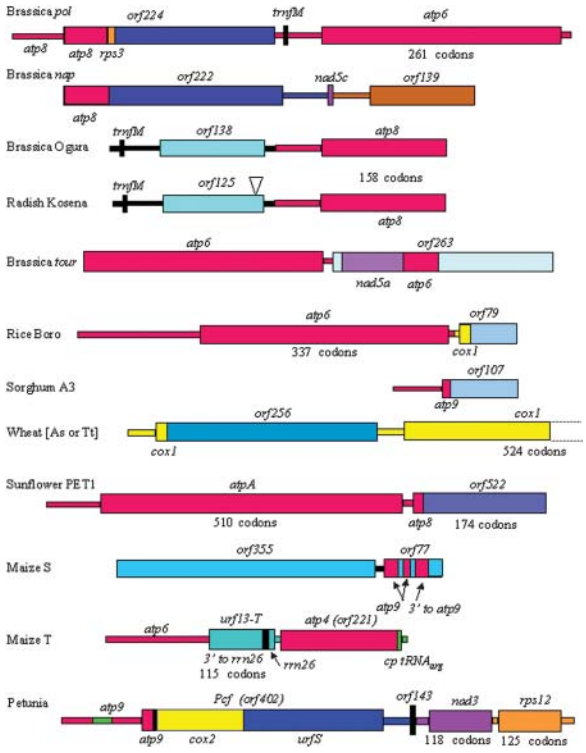

Figure 1. Chimeric Genes Associated with CMS.

*Orf*s are listed by the current convention of number of codons, except for loci named otherwise to be consistent with historical convention (*urf13* encodes a 13-kD protein; *pcf* indicates petunia CMS-associated fused gene; *orf522* encodes 522 nucleotides rather than 522 codons). References are cited in the text. Red indicates genes for subunits of ATP synthase. Shades of blue indicate unknown reading frames within CMS-associated regions. Shades of yellow indicate genes for subunits of cytochrome oxidase. Orange indicates ribosomal protein genes. Shades of brown indicate conserved unidentified reading frames found in multiple vascular plant mtDNAs. Green indicates chloroplast-derived sequences. Additional details of the sequences can be found in references cited in the text.

disrupted the *urf13* gene (Umbeck and Gengenbach, 1983; Abbott and Fauron, 1986; Rottmann et al., 1987). A particularly important revertant was described by Wise et al. (1987), who observed that a 5-bp insertion in *urf13*, causing a frame shift and a premature stop codon, was sufficient to confer fertility. Analyses at the RNA (Kennell et al., 1987; Kennell and Pring, 1989) and protein (Dewey et al., 1987; Wise et al., 1987) levels showed that *Rf1* affects the expression of *urf13* but that *Rf2* does not, even though both dominant alleles are required to restore fertility. The lack of an effect of *Rf2* on gene expression is understood, now that *Rf2* has been cloned and identified as an aldehyde dehydrogenase (Cui et al., 1996); this point is discussed further below.

The next CMS-associated locus to be identified contained completely different sequences than maize *urf13-T* but also contained a chimeric gene that clearly arose from a series of recombination events. In *Petunia*, a restriction fragment length polymorphism was identified that segregated with CMS in somatic hybrids containing recombinant mtDNAs (Boeshore et al., 1985), leading to the finding of a CMS-associated locus containing the so-called *pcf* (petunia CMS-associated fused) gene (Figure 1), which is composed of portions of the coding regions of *atp9*, *cox2*, and an unidentified reading frame termed *urfS* (Young and Hanson, 1987). The locus also contains the normal copies of the *nad3* and *rps12* genes as well as nine codons evidently derived from an unidentified gene, *orf143*, found in fertile petunia lines (Hanson et al., 1999). The second crucial type of evidence implicating this locus as causal in CMS was the detection of altered gene expression in the presence of the single dominant *Rf* allele. One class of transcripts, those that terminate at –121 before the *pcf* gene, are reduced in restored lines (Young and Hanson, 1987), and the abundance of the protein products of the *pcf* gene is greatly reduced (Nivison and Hanson, 1989). *pcf* encodes a 45-kD protein that is processed to a 19.5-kD protein that exhibits a mobility of 25 kD on acrylamide gels (Nivison and Hanson, 1989; Nivison et al., 1994). The larger precursor protein appears to be efficiently processed and is present in much lower quantities than is the 19.5-kD protein.

The identification of maize *urf13* and petunia *pcf* established two important features of CMS-associated loci. First, both loci arose from recombination events that created novel ORFs. Second, the same progenitor reading frame was not found in the two genes (Hanson et al., 1989). This was perhaps fortunate, for if there had been similarity between the two chimeric genes, erroneous searches for homologous genes in CMS genotypes of other species may have ensued.

ATP Synthase Subunit Sequences and Genes Often Are Found in CMS-Associated Loci

Now that ~12 mtDNA regions associated with CMS have been identified, it is striking how often the events involve ATP synthase subunit gene promoter regions and portions of coding regions (Figure 1). Even if the chimeric gene itself does not involve ATP synthase sequences, often the *orf* is located near an *atp* gene. In petunia, the N-terminal region of the *atp9* subunit is present in the CMS-associated *pcf* gene. In maize, not only does the *atp6* gene provide the 5′ regulatory sequences for the *urf13-T* gene, but the linked and cotranscribed *orf221* gene was implicated recently as a protein of the F_o subunit of ATP synthase. When the Arabidopsis ATP synthase complex was subjected to proteomic analysis, a protein similar to maize *orf221* was found to be present in the F_o portion of the complex and to exhibit some features characteristic of the b subunits of ATPases from various organisms (Heazlewood et al., 2003). For purposes of this review, *orf221*-homologous genes are referred to as *atp4*, the nomenclature used for the comparable yeast gene.

ATP Synthase Subunit 4, 6, 8, and 9 Coding Sequences in CMS-Associated Loci

A 159-codon sunflower gene has been shown to encode ATPase subunit 8 (Sabar et al., 2003). Sequences highly similar to this gene are found in many other plant mitochondrial genomes and have been termed *orfB* in previous reports. Furthermore,

a protein with a sequence similar to that of ORFB was identified as ATP8 in purified Arabidopsis ATP synthase (Heazlewood et al., 2003). As a result, such genes are referred to here as *atp8*. The *atp8* gene features prominently in CMS in *Brassica*, *Raphanus*, and sunflower.

Two different tactics were followed to identify the CMS-associated region in the mtDNA of the radish Ogura cytoplasm. Restriction maps of the 242- and 257-kb genomes of CMS and normal radish, respectively, were produced, but despite this prodigious effort for the time, a CMS-associated locus was not identified, although novel rearrangements between the two mtDNAs were detected (Makaroff and Palmer, 1988; Makaroff et al., 1989). Instead, the recombinational genetics strategy previously used in *Petunia* led to the identification of the Ogura CMS-associated locus. Examination of fertile and sterile cybrids (containing the nuclear genome from *Brassica* but the radish cytoplasm) revealed an Ogura cytoplasm mtDNA region that was correlated with CMS composed of a unique transcribed gene termed *orf138* next to the *atp8* gene (Bonhomme et al., 1991, 1992). Unstable cybrids gave rise to fertile progeny in which the *orf138* gene was lost or not transcribed (Bonhomme et al., 1992), providing strong evidence for its role in pollen abortion. The fertile revertant cybrids also lacked the 19-kD ORF138 protein detected in sterile lines (Grelon et al., 1994). The ORF138 protein product also is reduced greatly in fertility-restored lines containing *Rfo* (Grelon et al., 1994; Krishnasamy and Makaroff, 1994; Bellaoui et al., 1999); however, no difference in the *orf138* transcript profile was detected in fertile versus CMS plants (Krishnasamy and Makaroff, 1993; Bellaoui et al., 1999). When the region homologous with *orf138*/*atp8* in Kosena radish was sequenced, a gene highly similar to *orf138* was detected next to *atp8*. The Kosena CMS-associated gene *orf125* lacks 39 nucleotides found in *orf138* and encodes only 125 residues (Figure 1). An altered transcript profile in restored lines was not detected, but the ORF125 protein was observed not to accumulate in plants carrying *Rfo* (Iwabuchi et al., 1999).

ATP synthase genes also figure in the CMS-associated regions of two other *Brassica* cytoplasms, *pol* and *nap*. Comparative genomic analysis was informative in identifying the locus, largely because the mtDNAs of the normal and *pol* cytoplasm exhibit only one major rearrangement (L'Homme and Brown, 1993). Differences in mtDNA transcript sizes between CMS and fertile lines were detected by hybridizing probes for 14 known mitochondrial genes to RNA gel blots. Observation of altered *atp6* transcripts focused attention on the region surrounding the gene, where a cotranscribed upstream-unique gene was found. This chimeric gene, *orf224*, is composed of the first 58 codons of *atp8* fused to ~13 codons of *rps3* and then fused to unidentified sequences (Singh and Brown, 1991; Handa et al., 1995). The presence of the *Rfp* allele increased the proportion of monomeric *atp6* transcripts, thus implicating the region in CMS (Singh and Brown, 1991).

The high degree of similarity between the *nap* and *pol* mtDNAs suggested that close examination of the region in the *nap* mtDNA homologous with the *pol* CMS-associated region might be informative. Sequencing of the corresponding region in the *nap* CMS mtDNA resulted in the identification of a chimeric gene termed *orf222*, which, although downstream of *atp6*, is not cotranscribed with *atp6*. Instead, *orf222* is transcribed with the tiny exon c of *trans*-spliced *nad5* and a gene termed *orf139* (Figure 1). The identity of *orf139* is not known, although sequences similar to those of the gene are present in other mitochondrial genomes. Following the N-terminal 58-codon region similar to *atp8*, the protein sequences of the remainder of the *orf222* and *orf224* genes are 75% identical. The *orf222*/*nad5c*/*orf139* transcript profile is affected by the presence of the *Rfn* allele, providing a correlation to CMS (Brown, 1999).

Although most molecular analysis of *Brassica* CMS lines has occurred with the *nap*, *pol*, and Ogura cytoplasms, two additional CMS cytoplasms have been examined for the presence of aberrant mitochondrial genes. When *Brassica tournefortii* cytoplasm is introgressed into either *B. napus* or *B. juncea*, the lines become CMS. Probing of RNA gel blots from *B. napus (tour)* CMS versus restored lines with 17 gene-specific probes revealed a longer *atp6*-hybridizing transcript in CMS versus restored lines (Landgren et al., 1996). Sequencing of the region near *atp6* revealed a 263-codon *orf* gene containing ~90 codons similar to *nad5* exon a, followed by a 45-codon segment with similarity to a portion of the *atp6* coding region (Figure 1). A 32-kD protein was observed in CMS but not in restored lines upon in organello protein synthesis (Landgren et al., 1996). Whether the *orf263* gene encodes the 32-kD protein is not known.

Mitochondrial genes of the Tournefortii-Stiewe CMS cytoplasm, created by protoplast fusion between *B. tournefortii* and *B. napus*, also have been characterized. Mitochondrial RNAs that encode 25 mitochondrial genes in CMS and restored lines were screened for differences in expression level and transcript pattern. Altered transcript profiles led to the identification of a region containing a chimeric gene, *orf193*, carrying sequence similar to that of *atp6* next to an *atp9* gene. The protein products of this locus are not yet characterized, but they could include a chimeric protein encoded by both the *orf193* and *atp9* coding regions, because *orf193* and *atp9* sequences together can form one large ORF of 267 codons (Dieterich et al., 2003).

The *atp8* coding region also is found in a sunflower CMS-associated region (Figure 1). Progeny of an interspecific cross between *Helianthus petiolaris* and *H. annuus* (Leclerq, 1969) gave rise to male-sterile genotypes. A CMS cytoplasm arising from Leclercq's experiments, termed PET-1, is the most widely used and best characterized. Complete mitochondrial genome maps of the mtDNA in CMS and fertile plants revealed a single region near *atpA* that differed in arrangement between mtDNA in the CMS plant and that of *H. annuus.* The transcript profile of *atpA* transcripts differed between the two lines and also was affected by the presence of a fertility restorer (Siculella and Palmer, 1988). Following this lead, the region around *atpA* was characterized further, leading to the discovery of a novel ORF that encodes 522 nucleotides (174 codons) downstream of *atpA* (Kohler et al., 1991; Laver et al., 1991). The first 19 amino acids of ORF522 are identical to those of ATP8 (Sabar et al., 2003). In restored lines, transcripts of the novel *orf* gene are reduced in florets but not in seedlings (Moneger et al., 1994). This reading frame, termed *orf522* by the investigators, encodes a 15- to 16-kD protein detectable in flowers of CMS but not restored lines (Horn et al., 1991; Moneger et al., 1994).

atp9 is the ATP synthase gene implicated in the maize S cytoplasm. Unlike CMS-T and most other well-characterized CMS systems, fertility restoration in maize CMS-S is gametophytic. Pollen carrying the *rf3* allele are abnormal and sterile, whereas pollen carrying *Rf3* are fertile. Another uncommon feature of CMS-S is that cytoplasmic revertants occur relatively frequently in field-grown populations. Analysis of rearranged mtDNAs in revertant lines focused attention on a region containing two ORFs, *orf355* and *orf77*, whose transcript profiles are affected by the presence of the *Rf3* allele (Zabala et al., 1997). The pollen *orf77* transcript population is incompletely edited at a site that causes a premature stop codon, potentially resulting in the synthesis of a 17–amino acid polypeptide (ORF17) as well as the 77-residue protein predicted from unedited transcripts (Gallagher et al., 2002). The origin of the *orf355* gene is unknown, whereas *orf77* is a chimeric gene that contains portions of the coding region of *atp9* (Figure 1). ORF17 is composed primarily of residues from the C-terminal ATP9 domain (Gallagher et al., 2002). Protein products of the *orf355*/*orf77* region have not been identified.

Like maize CMS-S, fertility restoration of the sorghum A3 cytoplasm is gametophytic. A chimeric gene termed *orf107* predicts a protein carrying an N-terminal region related to the N terminus of ATP9 (Tang et al., 1996, 1999). The *atp9*-homologous region of sorghum *orf107* has 23 of 31 residues identical to those of *atp9*, followed by a region in which 28 of 51 codons predict amino acids identical to those of the CMS-associated rice *orf79* (Figure 1). The *orf107* transcript profile in lines carrying the *Rf3* allele differs from that in CMS lines. This apparent RNA processing event is correlated with sterility, but an earlier finding of altered RNA editing was not replicated when field-grown sorghum plants were examined (Pring and Van Tang, 2001). Whether an ORF107 protein accumulates differentially in CMS and restored lines is not known. The gene in the *Rf3* locus responsible for *orf107* transcript processing appears to be closely linked to a gene that encodes the processing of transcripts of an ORF of 209 codons, now known to be *atp4*. However, *atp4* (*orf209*) processing also occurs in nonrestoring lines (Tang et al., 1999).

Cytochrome Oxidase Subunit Gene Coding Regions in CMS-Associated Loci

The fusion of *atp9* and *cox2* coding regions in the *Petunia pcf* genes could be traced to short similar sequences present in the coding regions of the wild-type versions of the two genes (Pruitt and Hanson, 1991b). Short regions of similarity have also been shown to be important in abnormal recombination events that give rise to the maize nonchromosomal stripe mutants, which have mtDNA deletions (Newton, 1993). Thorough characterization of the recombination events of other chimeric genes may give similar clues to the origin of the novel aberrant genes.

coxI apparently has participated in recombination events in two characterized monocot CMS-associated regions. In wheat, most is known at the molecular level about the CMS types arising from crossing either *Aegilops speltoides* or related *Triticum timopheevi* cytoplasm into a *T. aestivum* nuclear background. Such lines can be restored to fertility by introducing nuclear genes from *T. timopheevi*. Both of the CMS-inducing cytoplasms contain a novel chimeric gene termed *orf256*, which has *coxI* sequences at its N terminus and is cotranscribed with an apparently normal *cox1* gene. Antisera prepared against polypeptide sequences predicted from *orf256* recognized a 7-kD protein present in the CMS line but not in the parental or restored lines (Song and Hedgcoth, 1994; Hedgcoth et al., 2002).

cox1 also is implicated in one type of rice CMS. Although several different CMS cytoplasms are used for the production of hybrid rice seed, only one of these has been characterized extensively at the molecular level, the ''Boro'' CMS, which is restored to fertility by a gene termed *Rf-1*. Cybrid plants containing recombinant mitochondrial genomes were analyzed to reveal that a region downstream of *atp6* was correlated with CMS (Akagi et al., 1995). The Boro CMS cytoplasm contains an ORF of 79 codons, termed *orf79*, which is similar to *coxI* at its N terminus (Figure 1). Of the first 28 predicted amino acids of ORF79, 19 are identical to those of rice *cox1* as described in the complete rice mtDNA sequence (Notsu et al., 2002). Of particular interest is the 80% similarity of the remainder of ORF79 to the unidentified reading frame in sorghum A3 cytoplasm (Tang et al., 1999). The sizes of transcripts in this region are altered in the presence of *Rf-1* (Akagi et al., 1994), and the presence of *Rf-1* affects the editing extent of *atp6* transcripts, perhaps indirectly as a result of RNA processing (Iwabuchi et al., 1993). Because the protein products of *atp6* and *orf79* in the rice Boro CMS line have not been characterized, it is not known whether their sequence or abundance is altered in fertility-restored lines.

Unique Unidentified Sequences Found in CMS-Associated Loci

The prior discussion has highlighted the known coding regions that have participated in recombination events to produce novel genes and operons in CMS cytoplasms. A feature common to almost all characterized CMS-associated regions is the involvement of unidentified sequences that have no similarity to conserved plant mtDNA sequences (Figure 1). The origin of these *orf*s remains a mystery, because they have no detectable significant similarity to chloroplast or plant nuclear sequences, despite the availability of completely sequenced genomes.

In one characterized species, the coding region of the CMS-associated gene is composed entirely of sequences of unidentified origin. A CMS-associated region was identified in bean by comparing CMS lines and fertile lines arising as revertants (Mackenzie and Chase, 1990). This region contained a unique mtDNA region that was termed *pvs* (*Phaseolus vulgaris* sterility) and was found to contain a 239-codon *orf* gene. The same region is gradually lost in subsequent generations after crossing in the *Fr* locus, providing a second correlation with CMS (He et al., 1995). Furthermore, another restorer locus, *Fr2*, which does not affect mtDNA composition, affects the transcript profile of the *pvs* region (Chase, 1994). The protein product of *orf239* was found to accumulate only in reproductive tissues of CMS plants (Abad et al., 1995; Sarria et al., 1998). ORF239 was further implicated as the cause of pollen disruption in common bean when tobacco plants expressing the gene from a nuclear transgene were found to be male sterile (He et al., 1996). Similar

transgenic experiments with other CMS-associated genes from other species, such as maize and petunia (von Allmen et al., 1991; Chaumont et al., 1995; Wintz et al., 1995), have not resulted in male-sterile plants, perhaps because the transgene's expression or the transgenic protein's mitochondrial targeting did not adequately mimic the expression of the genuine mitochondrial gene. The experiments with *pvs* may have succeeded in producing CMS plants because, remarkably, the gene product is found external to the mitochondria, associated with the cell walls of developing pollen (He et al., 1996).

MECHANISMS OF ACTION OF CMS-ASSOCIATED GENES

The precise action of a mitochondrial locus that confers male sterility has not been determined definitively for any species. One technical problem that plagues many attempts to compare reproductive tissue in CMS and fertile lines is obtaining sufficient quantities of reproductive tissue for physiological and biochemical studies. Most such studies have been performed on vegetative tissues that express the CMS-associated proteins; however, whether the same disruption occurs in the tapetal layer or young microspores remains a subject for further investigation in many species. Nevertheless, biochemical and physiological studies have provided some clues to the disruption that results in aborted pollen development.

The URF13 Protein of Maize CMS-T

The maize CMS-T URF13 protein has been subjected to intense scrutiny because it encodes not only CMS but also sensitivity to T-toxin produced by race T of the fungus *Cochliobolus heterostrophus* and to the insecticide methomyl. In the presence of the toxin, URF13 forms a pore in the inner mitochondrial membrane (Rhoads et al., 1995). Expressing URF13 from the nuclear genome and targeting it to the tobacco mitochondrion caused transgenic plants to be sensitive to both T-toxin and methomyl, although the plants were not male sterile (von Allmen et al., 1991), perhaps because of the use of the 35S promoter, which is poorly expressed in sporogenous tissue. Whether URF13 forms a similar pore in developing anthers, leading to pollen abortion, is not known. URF13 is expressed in vegetative tissue as well, but only pollen is disturbed. A hypothesis made by Flavell (1974) remains an intriguing possibility: perhaps some biosynthetic product found only in young anthers interacts with URF13 in a manner analogous to methomyl or T-toxin, leading to pore formation and cell death.

Possible Disruption of Mitochondrial Membranes

Inspection of the sequences of chimeric genes associated with CMS (Figure 1) has shown that many of them carry predicted transmembrane domains, and a number of the encoded proteins have been shown experimentally to be loosely associated with, or integrated into, the inner mitochondrial membrane. However, whether the proteins disrupt a particular complex within the membrane, or perhaps create a pore, as suggested by the URF13 studies, is not known. Various mitochondrial functions have been compared between tissues of CMS and fertile lines of a number of different species (reviewed by Conley and Hanson, 1995). For example, differences in alternative oxidase activities have been detected in tissue from *Petunia* and *Nicotiana* CMS lines (Sabar et al., 2000; A. Moore, unpublished data), but these alterations could result from a primary disruption in some other pathway. Enhanced alternative oxidase expression in certain *Nicotiana* CMS lines appears to result from the dysfunction of the mitochondrial NADH dehydrogenase (Gutierres et al., 1997). Although these and other studies have revealed differences in the level of activity of various respiratory complexes between CMS and fertile tissues, whether these differences are the primary cause or merely effects of other mitochondrial defects is not known.

An Effect on ATP Synthase Function?

Consideration of the CMS-associated chimeric genes shown in Figure 1 reveals that many of them include portions of ATP synthase subunits, whereas others are closely linked to apparently normal ATP synthase subunit genes. This observation raises the possibility that impaired ATP synthase activity could be a causal factor in the disrupted pollen development in CMS lines in a number of species. An alloplasmic CMS *Nicotiana* genotype was found to have a lower ATP:ADP ratio in floral buds than normal tobacco (Bergman et al., 2000). Recently, Sabar et al. (2003) found that mitochondrial ATP synthase in seedling tissue from sunflower CMS lines was lower than that in restored lines, according to in-gel enzymatic assays. Similar assays of NADH dehydrogenase, succinate dehydrogenase, and cytochrome oxidase did not reveal any activity differences.

Is the Expression of Linked Genes Affected?

Most attention has been focused on the active disruption of a mitochondrial function by the expression of an unknown or chimeric *orf*. However, for at least some of the characterized CMS systems, it remains possible that the presence of the *orf* causes impairment of the expression of a closely linked mitochondrial gene that has a normal coding region. In this scenario, the downregulation, processing, or destabilization of transcripts and proteins of the CMS-associated region found in restored lines does not rescue pollen development by removal of a toxic protein; instead, it results in normalized expression of the linked genuine mitochondrial gene. There are a number of examples of RNA processing in restored lines of various species that results in the production of a monocistronic transcript of the linked mitochondrial gene. In a few CMS systems that feature essential genes within the CMS-associated region, the expression of a closely linked gene, such as petunia *rps12* and maize *atp4* (Prioli et al., 1993; Lu et al., 1996), has been examined at the protein level and found not to be abnormal. Similar analyses of the expression of the genes shown in many of the CMS-associated regions in Figure 1 wait to be performed.

A Special Role for Mitochondria in Pollen Development?

A long-standing mystery regarding CMS is why an abnormal mtDNA region should specifically disrupt pollen development. In some systems, such as common bean, the explanation is

relatively straightforward: the aberrant protein is expressed only in reproductive tissue. In common bean, the CMS-associated gene product is apparently degraded by a protease in mitochondria of vegetative tissue (Sarria et al., 1998). A second explanation for pollen specificity could be increased levels of a toxic protein in tapetal tissue or sporogenous tissue. Expression of wild-type mitochondrial proteins and mitochondrial transcripts varies between different tissues in the plant (Conley and Hanson, 1994; Moneger et al., 1994), so aberrant proteins could possibly change in concentration during anther development. However, it is technically difficult to distinguish whether the concentration of a protein within a mitochondrion has changed in young anthers or whether apparently enhanced levels of a mitochondrial protein are an indirect result of an increase in mitochondrial number, which has been documented in the tapetum and wild-type sporogenous tissue of maize (Warmke and Lee, 1978). Perhaps the leading hypothesis for most types of CMS is that mitochondria have a special role to play in the tapetum, male meiotic cells, and/or developing microspores. At this stage in development, demand for energy or particular mitochondrial biosynthetic products may be especially high, so that impairment of mitochondrial function becomes devastating. Consistent with this hypothesis is the finding that antisense downregulation of alternative oxidase or pyruvate dehydrogenase in the tobacco tapetum resulted in microspore death (Yui et al., 2003). Furthermore, although *N. sylvestris* plants that contain a deletion in a gene that encodes a mitochondrial NADH dehydrogenase subunit survive, they are male sterile (Gutierres et al., 1997).

Pollen Abortion by Programmed Cell Death

Whatever defect in mitochondrial function is caused by a CMS-associated region, its ultimate result may be programmed cell death (PCD) of either the tapetal layer or sporogenous cells. Before PCD was recognized as a specific phenomenon, many investigators documented microscopic evidence of premature breakdown of the tapetal layer and mitochondrial morphology changes in dying anther cells (reviewed by Laser and Lersten, 1972; Kaul, 1988). As in animals, plant mitochondria appear to play a role in interpreting signals for cell death (Lam et al., 2001). Balk and Leaver (2001) provided evidence that tapetal cells in sunflower CMS lines exhibit characteristic features of PCD, including cytochrome *c* release. It is likely that PCD will be found to occur in CMS lines of many other species when the dying cells are examined closely.

EFFECTS OF CMS-ENCODING GENES ON FLORAL MORPHOLOGY

CMS lines of many species exhibit perfectly normal floral morphology; the only obvious difference between flowers of fertile and CMS lines is the absence or presence of pollen or fully developed anthers (see petunia, sunflower, and maize examples in Figure 2). In other species, however, male sterility is accompanied by apparent homeotic alterations in floral tissue identity. These changes often have been noted by plant breeders who have performed interspecific crosses. For example, the alloplasmic wheat line containing the *orf156* locus (Figure 1)

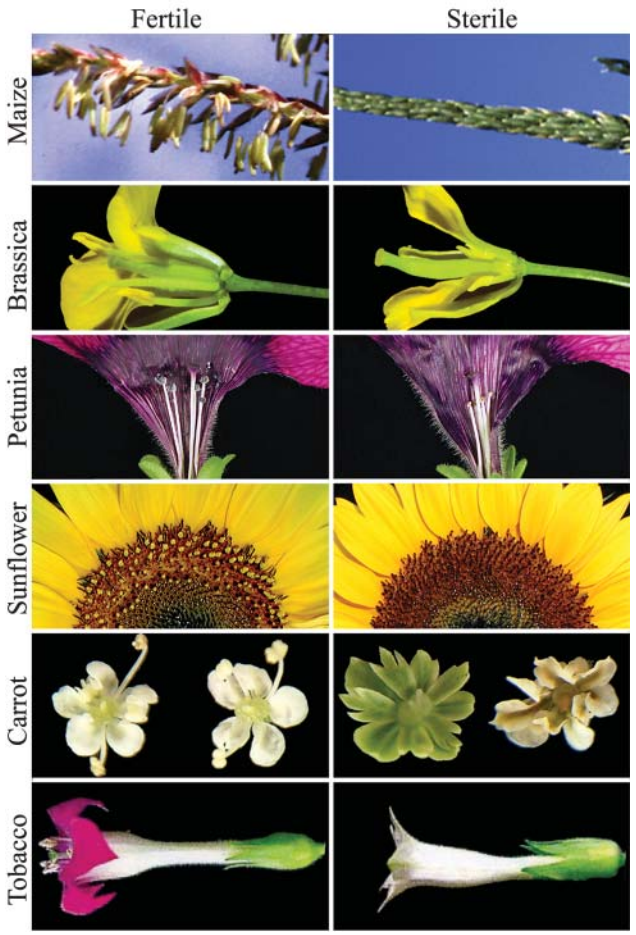

Figure 2. Floral Morphology in CMS and Fertile Plants.

For maize, fertile lines exhibit exserted anthers, unlike the CMS-T line. For *Brassica*, flowers containing Ogura cytoplasm exhibit altered stamen morphology. For petunia, CMS and fertile flowers are indistinguishable except for degenerating anthers and a lack of pollen in the sterile line. For sunflower, flowers in CMS lack the pollen evident on the wild-type flower. For carrot, stamens in CMS have been converted to petal- or bract-like structures. For tobacco, CMS flowers have no exserted stamens, which are fused with carpels. Photographs courtesy of G. Brown (*Brassica*), P. Simon (carrot), R. Wise (maize), and K. Glimelius (tobacco).

exhibits pistillody, the transformation of stamens into pistil-like structures, and fertility restoration results in normal flowers as well as viable pollen. The radish Ogura CMS cytoplasm in the *B. napus* background results in altered floral morphology (Figure 2). Individual cybrid lines containing the *B. napus* nuclear genome and recombinant mtDNA derived from a combination of *B. napus* and Arabidopsis exhibited homeotic transformation of anthers to carpels and some disturbances in vegetative growth (Leino et al., 2003). In carrot, lines containing a CMS cytoplasm may exhibit either a petaloid (Figure 2) or a carpeloid phenotype, depending on nuclear background. Floral morphology also is disturbed when crosses or cybrids are made between different *Nicotiana* species (Gerstel et al., 1978; Nikova and Vladova, 2002), such as introducing the *N. repanda* cytoplasmic genomes into

a *N. tabacum* nuclear background (Figure 2). Interspecific cybrids between *N. tabacum* and *Hyoscyamus niger* containing recombinant mitochondrial genomes also resulted in individuals with a variety of floral modifications (Zubko et al., 2003).

Several investigators have recognized that the floral homeotic alterations found in these alloplasmic CMS lines resemble the mutants in *Antirrhinum* and Arabidopsis, whose analysis resulted in the synthesis of a model for floral patterning (Weigel and Meyerowitz, 1994). Accordingly, the expression of homologs of the known floral homeotic genes has been analyzed in deformed CMS flowers versus wild-type or restored lines. In wheat, transcripts of the APETALA3 homolog were reduced in pistilate florets (Murai et al., 2002). In carpeloid carrot flowers, reduced expression of homologs of *Antirrhinum* GLOBOSA and DEFICIENS were detected (Linke et al., 2003). The first direct test of the hypothesis that disturbed expression of the known floral homeotic genes could be responsible for CMS-associated abnormal floral morphology was performed by Bereterbide et al. (2002). They transformed a *N. tabacum* CMS line with the tobacco homolog of Arabidopsis SUPERMAN. Flowers in the CMS line (Figure 2) had severely distorted stamens fused to the carpel, but transgenic plants exhibited flowers with improved morphology, including free stamens and some functional pollen (Bereterbide et al., 2002). How particular mitochondrial/nuclear gene combinations can produce such disturbances in the normal floral developmental pathway is an intriguing topic for further inquiry.

MECHANISM OF ACTION OF RESTORER LOCI

Although restorer alleles are known to affect all of the well-characterized CMS-associated genes in the species described above, the mechanism of action has not been determined definitively for any restorer. With the exception of maize *Rf2*, all restorers are known to affect either the transcript profile or the protein accumulation of the CMS-associated locus, and some have been observed to affect both RNA and protein products.

A complication in discerning the mode of action of a restorer locus that affects a transcript profile is the complex transcript population often found for a particular plant mitochondrial gene. Many such genes exhibit multiple transcript initiation and processing sites. Termini must be analyzed with capping enzyme, which labels termini arising from initiation but not by processing, to determine how the transcript was produced.

Mitochondrial RNA Processing Events Affected by Fertility Restoration

Analysis of the transcripts of the *Brassica nap* and *pol* CMS-associated region has revealed that *Rfp* results in enhanced processing of a dicistronic *orf224/atp6* transcript so that two monocistronic *atp6* transcripts increase in abundance (Li et al., 1998; Brown, 1999). This result implicates *Rfp* in RNA processing, but it is consistent with both the removal of a detrimental action of *orf224* and a possible improved expression of *atp6* from a monocistronic transcript. Genetic analysis indicates that *Rfp* and *Rfn* are allelic, with *Rfn* likely involved in processing of the *orf222/nad5c/orf139* locus. In addition, the presence of *Rfn* changes the transcript profile of *nad4* and *ccl1* (Singh et al., 1996). Based on these observation, Brown and colleagues have suggested that the *Rfn/Rfp* locus may be complex, containing several closely linked genes (Li et al., 1998). Recent discoveries about restorer loci made through map-based cloning (discussed below) are consistent with this hypothesis.

Another example of a restorer locus affecting more than one transcript comes from the CMS-S system in maize. The *Rf3* locus segregated with the presence of shorter transcripts of not only the CMS-associated *orf355/orf77* region but also smaller transcripts of *cob* and *atp6* compared with fertile lines (Wen and Chase, 1999a). The effect on multiple transcripts could be the result of the action of the same RNA processing enzyme (e.g., on several different transcripts), or perhaps the *Rf3* locus consists of two or more closely linked genes that act on different RNA substrates.

Processing of transcripts of the maize CMS-T *urf13* gene has been shown to be correlated with the *Rf1* locus. In addition to *Rf1*, the presence of either of two additional genes that confer partial fertility restoration, *Rf* and *Rf**, has been correlated with the processing of *urf13* transcripts (Dill et al., 1997). Processing events in the presence of each of the restorers occur in different locations within *urf13* RNAs; interestingly, the processing sites exhibit some sequence conservation. What remains puzzling, however, is that although the processing events produce transcripts from which URF13 cannot be translated, the restored lines still contain larger transcripts that could encode URF13, with no obvious decrease in RNA abundance relative to CMS lines (Wise et al., 1999). A similar puzzling result has been seen in restored petunia lines, which contain transcripts that could encode the *pcf* gene product (PCF), even though transcripts with 5′ termini –121 to the start codon are decreased in abundance (Pruitt and Hanson, 1991a). Like maize URF in restored lines, it is not known why PCF is sharply reduced in abundance despite the presence of transcripts spanning the region.

Finding a difference in transcript profile does not necessarily mean that the restorer acts at the level of transcription or RNA processing. There is evidence for instability of the transcripts of the sunflower CMS-associated region in restored lines; the RNAs exhibited increased polyadenylation and enhanced degradation in male florets (Gagliardi and Leaver, 1999).

Restorers, Mitochondrial Translation, and Protein Stability

An alteration in transcript profile could be the indirect result of defective translation. In yeast mitochondrial mutants that lack a nucleus-encoded translation factor for a particular message, the mRNA is sometimes destabilized when it cannot be translated (Costanzo and Fox, 1990). One way to determine whether a transcript is being translated is to determine whether it is present on polysomes. Unfortunately, as discussed above, the mitochondrial disruption leading to CMS often occurs very early in pollen development, when anthers are very small and it is difficult to obtain quantities of tissue for biochemical analysis. Furthermore, the microscopic analysis of CMS plants in many species has indicated that disruption of mitochondrial integrity in

the tapetal layer is the first sign of abnormal development and thus may be the cause of microspore abortion (Laser and Lersten, 1972; Kaul, 1988). Because the tapetal layer represents only a small percentage of the total anther tissue, features of the expression of CMS-associated regions in CMS versus restored lines may not be reflected accurately by analysis of total anther transcripts and proteins. For such species, in situ methods may be necessary.

In *B. napus* CMS or restored lines, sufficient quantities of <3-mm anthers could be obtained to perform polysome analysis. Bellaoui et al. (1999) took advantage of a cybrid with an altered *orf138/atp8* region that results in the formation of separate mRNAs for *orf138* and *atp8*, unlike lines carrying the original Ogura cytoplasm, in which the two genes are cotranscribed. The *orf138* mRNA was found to sediment with polysomes extracted from anthers, indicating that translation could occur, but no ORF138 accumulated in floral buds of the restored cybrid line (Bellaoui et al., 1999). These results suggest that *Rfo* acts post-translationally to affect protein stability.

Fertility Restoration through mtDNA Alteration

Unlike all other restorer genes studied to date at the molecular level, the *Fr* locus in common bean results in the loss of a particular CMS-associated mtDNA region. No effect of a restorer locus on the quantity of such a mtDNA region has been detected in other species. Actually, the common bean *pvs* region is not lost entirely, but it is reduced to a substoichiometric level (Janska et al., 1998). Thus, *Fr* apparently reduces the expression of CMS-associated *orf239* by reducing the amount of the encoding DNA rather than the RNA or protein. Because the *Fr* locus has not yet been cloned, it is not known how it shifts the amount of this particular mtDNA. Little is known about how the organization of the mitochondrial genome is maintained and affected by nuclear gene products. Indeed, even the configuration of the vascular plant mitochondrial genome in vivo is not known. Restriction maps consistent with the existence of a ''master circle'' and usually a number of subgenomic circles arising from recombination have been produced for a number of species (reviewed by Hanson, 1991; Hanson and Folkerts, 1992). However, direct examination of mtDNA molecules revealed only a few large circular molecules among a mixture of large circular permutated linear molecules and concatemers (Oldenburg and Bendich, 2001).

A clue to the possible mode of action of *Fr* comes from the study of mutations in the Arabidopsis *chloroplast mutator* (*CHM*) locus, which result in the loss of mtDNA regions, producing green-white variegated leaves (Martinez-Zapater et al., 1992). Cloning of *CHM* revealed that it encodes a gene homologous with *Escherichia coli MutS*, which is involved in mismatch repair and DNA recombination (Abdelnoor et al., 2003). The investigators have proposed that *CHM* may be required for recombination or replication and, therefore, that mutation could result in modulation of the copy number of particular molecules (Mackenzie, 2004). Perhaps *Fr* affects such a process to alter the maintenance of molecules carrying the CMS-associated common bean DNA.

IDENTIFICATION OF RESTORER LOCI

Solving the question of the mode of action of restorer genes should be facilitated by their cloning and sequencing. Once a restorer gene is identified, a transgenic line can be created that is completely isonuclear with a sterile line except for the presence of a single restoring gene, providing ideal material with which to compare biochemical functions between tissues of CMS and restored lines. The functions of the restorer genes cloned to date are not obvious from sequence inspection alone, with the exception of maize *Rf2*.

Maize *Rf2* Encodes an Aldehyde Dehydrogenase

The first nuclear fertility restorer gene to be cloned was *Rf2* of maize, which acts in concert with *Rf1* to restore fertility to CMS-T maize. The gene, cloned by transposon mutagenesis, was immediately observed to exhibit similarity to aldehyde dehydrogenases (Cui et al., 1996). Subsequent studies of the encoded protein revealed that it has the predicted enzymatic activity (Liu et al., 2001). *Rf2* is evidently a ''biochemical restorer,'' which presumably acts by ameliorating a residual defect that remains when *Rf1* downregulates *urf13* expression. Plants of genotype *Rf2 Rf2*/*rf1 rf1* are sterile and exhibit no discernible difference in *urf13* expression from plants that are recessive at both restorer loci. Both *Rf1* and *Rf2* are required for normal pollen development in the CMS-T background. Very low levels of URF13 may cause a detrimental effect that can be compensated for by the mitochondrial aldehyde dehydrogenase. The role of *Rf2* could be to reduce the amount of a toxic aldehyde present as a result of residual URF13 expression. RF2 is capable of oxidizing both aliphatic and aromatic aldehydes; however, the identity of its substrate(s) in developing anthers is not known (Liu and Schnable, 2002).

Although all of the other restorer systems discussed in this review, which require only a single dominant allele, are known to have an effect on the expression of the CMS-associated mtDNA region, it is possible that some of the complex, multigenic restorer systems found in some species (reviewed by Laser and Lersten, 1972; Kaul, 1988) may include genes that act by rescuing the developing pollen through a metabolic effect, compensating for the presence of a toxic protein rather than preventing its expression.

Petunia CMS Lines Are Restored to Fertility by a Pentatricopeptide Motif Gene

The single dominant *Rf* gene of petunia was the first restorer allele cloned that is known to affect the expression of a CMS-associated mtDNA region. Molecular markers delimited a nuclear genomic region that segregates with *Rf* (Bentolila and Hanson, 2001), and testing of a candidate gene by transformation resulted in the identification of a gene that encodes a mitochondrially targeted protein of 592 residues that could restore fertility to transgenic plants that carry the petunia CMS cytoplasm (Bentolila et al., 2002). Not all *Rf-PPR592* transformants were fertile; restoration probably requires a threshold level of expression of the transgene (Alfonso et al., 2003). Progeny of

a primary fertile transformant exhibited segregation of the transgene with fertility and with greatly reduced abundance of the CMS-associated PCF protein. The restorer allele, termed *Rf-PPR592*, contains 14 copies of a pentatricopeptide repeat (PPR) motif, accounting for 87% of the coding region (Figure 3). Although transgenic plants of normal appearance were obtained when genomic DNA carrying *Rf-PPR592* was introduced (Bentolila et al., 2002), some transgenic plants exhibiting abnormal vegetative and floral development were obtained when the *Rf-PPR592* coding region was expressed under the control of the 35S promoter (A. Alfonso, S. Bentolila, and M.R. Hanson, unpublished data). Perhaps an analogous disturbance of development occurs in the alloplasmic CMS genotypes that have homeotic floral transformations (discussed above).

The petunia *Rf* locus is complex in that a second PPR gene (*Rf-PPR591*), encoding a 591–amino acid protein, is located in close proximity to *Rf-PPR592*. The function of *Rf-PPR591* is not known. A homologous gene was cloned from an *rf*/*rf* line and found to carry a deletion in the promoter region with respect to *Rf-PPR592* and to exhibit predicted amino acid sequence polymorphisms (Bentolila et al., 2002). This gene is not expressed in reproductive tissue, unlike *Rf-PPR592*.

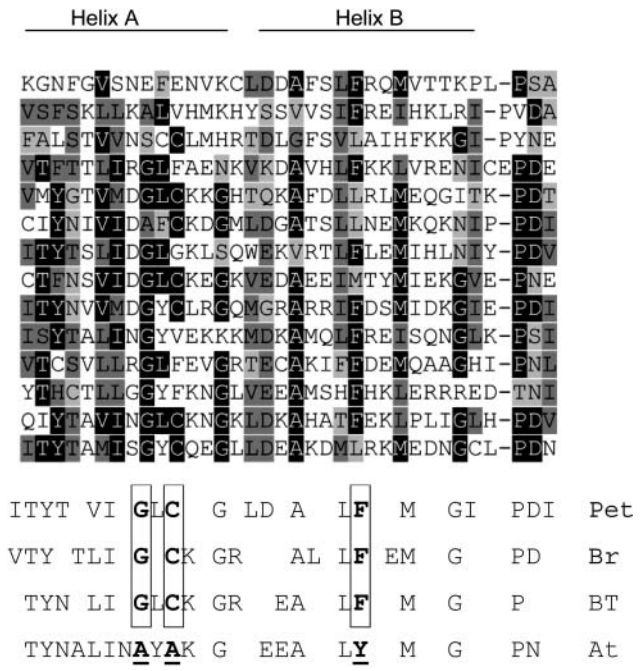

Figure 3. The PPR Motif and Nuclear Genes That Modify Mitochondrial Gene Expression.

(A) Alignment of the 14 motifs in the predicted petunia Rf protein. Each PPR was identified by MEME software (Bailey and Elkan, 1994). Identical amino acids are shown as black boxes with white lettering; at least 7 of the 14 motifs must have an identical or highly similar amino acid for the residue to be included in the petunia Rf consensus sequence. Similar amino acids are shown in dark gray and weakly similar residues are in light gray. The locations of the two predicted anti-parallel α-helices are indicated (Small and Peeters, 2000).

(B) Below the petunia Rf PPR motif alignment are shown the consensus sequences of the PPR motifs found in petunia Rf, *Brassica*/radish Rfo, and PPR8-1, the putative Boro rice restorer. The three Rf consensus sequences differ consistently at three residues (boxed) compared with those residues (underlined) in the consensus of 1303 PPR motifs analyzed in Arabidopsis by Small and Peeters (2000).

PPR Genes Also Are Found in *Brassica* and *Raphanus* Restorer Loci

The next restorer gene to be identified was the Ogura restorer found in *Brassica* and *Raphanus*. Three different groups have shown that the *Rfo* locus maps to a region that contains PPR motif–containing genes (Brown et al., 2003; Desloire et al., 2003; Imai et al., 2003; Koizuka et al., 2003). Map-based cloning of the *Rfk1* locus, which restores fertility to Kosena radish CMS lines, delimited the locus to two PPR genes. A gene we have termed *Rf-orf687* restored fertility to CMS *B. napus*, but the other PPR gene did not (Koizuka et al., 2003). Transgenic lines exhibited reduced abundance of the CMS-associated protein. A homologous gene cloned from a nonrestoring line exhibited a three-nucleotide substitution in the upstream region and a predicted protein with four amino acid differences relative to *Rf-orf687* (Koizuka et al., 2003). Brown et al. (2003) used synteny between Arabidopsis and radish to initially generate markers that narrowed the *Rfo* region to a 270-kb region, which was sequenced and found to contain 43 genes. Additional mapping further delimited *Rfo* to a 17-gene region. Fragments carrying each of these genes were introduced into the nuclear genome of Ogura CMS *B. napus* lines. A DNA region containing the same *Rf-orf687* gene identified in restored Kosena radish was able to confer fertility upon primary transformants and their progeny. Like Desloire et al. (2003), Brown et al. (2003) identified three PPR-containing genes mapping to the vicinity of the restorer locus. The other two PPR genes lack portions of repeats found in *Rf-orf687*, and evidently, neither was observed to restore fertility in transgenic plants (Brown et al., 2003). In summary, based on the reports from these groups, we conclude that the *Rfk1* and *Rfo* restoring alleles are identical and that a single ORF containing 16 PPR motifs is sufficient to restore fertility.

A PPR Gene Implicated in Boro Rice Restoration

A third nucleus-encoded PPR repeat gene has been shown to modify the expression of a CMS-associated region in rice, although whether this gene is actually a restorer is not yet known. The PPR gene identified by Kazama and Toriyama (2003) affects the transcripts of a CMS-associated gene present in the Boro cytoplasm. Restorer lines that confer fertility in rice lines carrying the Boro cytoplasm do not modify the male sterility of lines carrying the WA cytoplasm. The Boro CMS restorer is known to alter the expression of a CMS-associated region found in mtDNA from Boro CMS lines (Akagi et al., 1994). Kazama and Toriyama (2003) mapped the Boro CMS restorer *Rf-1* within 1 centimorgan and then searched for PPR genes carrying putative mitochondrial transit sequences within the vicinity, using the available rice genome sequence information. After transforming PPR genes

and examining the expression of the *atp6/orf79* region in callus, they observed that a gene termed *PPR8-1* results in transgenic callus with the same transcript profile as that found in restored Boro CMS plants (Kazama and Toriyama, 2003). These investigators used a strategy that was suggested as soon as the *Petunia Rf* locus was shown to be a PPR gene: map approximately to a genomic region, obtain genomic DNA sequence there, and then search for mitochondrially targeted PPR genes. Such genes can be tested either by transformation or by using the sequence information to generate markers for fine mapping of the gene to the restoring locus.

PPR-Containing Fertility Restorers Are Members of a Large Gene Family

The PPR motif was first described as a degenerate motif found in hundreds of genes in the available Arabidopsis nuclear genome sequence (Aubourg et al., 2000; Small and Peeters, 2000). Although much less numerous, PPR motif–containing genes also can be found in animal genomes. A consensus sequence was generated by computer analysis from 1303 35–amino acid repeats in Arabidopsis by Small and Peeters (2000) and is shown in Figure 3. The most frequently occurring amino acids at each position of the 14 petunia *Rf-PPR592* repeats and the 16 Ogura restorer *Rf-orf687* repeats are observed to contain four conserved residues that differ from the amino acids at the same position in the Arabidopsis consensus. Three of these four amino acids also are found when a similar analysis is performed on rice *PPR8-1* (Figure 3). The repeats in the petunia, Ogura restorer, and rice genes also exhibit a number of locations that adhere to the consensus described for the 1303 Arabidopsis repeats (Figure 3).

PPR proteins have previously been implicated in organelle gene expression in fungal mitochondria and in algal and plant chloroplasts (Small and Peeters, 2000). The yeast PET309 and the *Neurospora* CYA-5 PPR proteins are necessary for the maturation and/or accumulation of coxI mRNA and its translation (Coffin et al., 1997; Manthey et al., 1998). The maize *crp1* PPR-containing gene product is required for processing of the *petD* transcript and for the translation of *petA* and *petD* mRNAs (Fisk et al., 1999). Another maize PPR gene is required for the accumulation of plastid ribosomes (Williams and Barkan, 2003). A *Chlamydomonas* photosynthetic mutant in which the stability of the *petA* transcript is impaired has an alteration in the gene that encodes the PPR protein MCA1 (Lown et al., 2001). Mutants in the Arabidopsis *hcf152* PPR gene exhibit high chlorophyll autofluorescence and have defects in the processing and/or stabilization of transcripts of the *psbB-psbT-psbH-petB-petD* operon (Meierhoff et al., 2003). In the chloroplast stroma, maize CRP1 has been found in a large complex of unknown composition (Fisk et al., 1999), in contrast to Arabidopsis HCF152, which is not found in a high molecular mass complex (Meierhoff et al., 2003).

Whether the PPR proteins encoded by *Petunia Rf* and *Brassica Rfo* bind to particular RNAs or proteins is not yet known. A few PPR proteins have been shown to bind to RNA. HCF152 binds to *petB* transcripts (Meierhoff et al., 2003). A radish PPR protein of unknown function was identified in a screen for RNA binding proteins, although with which chloroplast RNA(s) it may normally interact is not known (Lahmy et al., 2000). Two animal PPR proteins, human LRP130 and *Drosophila* BSF, have also been shown to bind RNA (Mancebo et al., 2001; Mili and Pinol-Roma, 2003). Because the PPR repeat is quite similar to the tetratricopeptide repeat, which is known to form amphipathic α-helices and to bind proteins (Lamb et al., 1995), it is possible that some PPR proteins may bind proteins as well. Small and Peeters (2000) have proposed that PPR motifs may form a positively charged groove that could bind acidic proteins, phosphoproteins, or RNA. Identification of the ligands of PPR restorer proteins should help elucidate their function.

HOW DO CMS AND RESTORER SYSTEMS ARISE?

Nuclear fertility restorers are likely to have evolved from genes involved in the normal regulation of mitochondrial gene expression, a hypothesis consistent with prior molecular analysis as well as with the identification of restorer alleles as members of a large gene family. CMS-associated regions often appear to arise from illegitimate recombination events. Below, we speculate on how CMS and fertility restorers may arise and evolve in nature.

Plant mitochondrial genomes appear to be subject to relatively frequent successful invasions of DNA sequences from other genomes. Plant mtDNAs contain sequences obviously derived from chloroplast and nuclear genomes, as well as considerable sequences of unknown origin. Analysis of the complete sequences of the mitochondrial genomes from three vascular plants shows that they carry a complement of $\sim$55 to 60 genes that encode either known mitochondrial genes or conserved mitochondrial *orf*s of unknown function (Unseld et al., 1997; Kubo et al., 2000; Notsu et al., 2002). Strikingly, the remainder of the DNA in each of the sequenced genomes contains many sequences and *orf*s not found in either of the other two genomes. The propensity for recombination that is characteristic of plant mtDNAs may allow the integration of nucleic acids that happen to enter the mitochondrion. When an immigrant sequence lands next to gene regulatory sequences within mtDNA or fuses with a portion of an extra copy of a genuine mitochondrial gene, the novel sequence may become expressed. Perhaps sometimes an immigrant sequence enters the genome in an innocuous position where it is expressed not at all or at a low level. But a recombination event may occur so that the sequence enters a region more favorable for expression. Furthermore, the invading sequence may remain relatively quiescent, present only at a low copy number in substoichiometric amounts, until some alteration in the nuclear genes that control mtDNA replication and recombination induce an increase in the amount of the recombinant molecule. Evidence for this feature of our model comes from studies of *Phaseolus*. Arrieta-Montiel et al. (2001) were able to find the common bean CMS-associated *pvs* region in undomesticated *Phaseolus* lines and showed that 90% of the lines maintained the sequence at substoichiometric levels, whereas in other lines, the *pvs* region had increased as much as 2000-fold.

If expression of the invading sequence is detrimental to the plant, there will be selection pressure to downregulate its expression. However, if the novel sequence uses the same mitochondrial gene regulatory signals as genes for important mitochondrial functions, then expression of the invader must be reduced without preventing the expression of essential genes. One solution is the duplication of a nuclear gene that regulates RNA processing, translation, or RNA or protein stability within the mitochondrion, followed by divergence of one copy to act specifically on the products of the novel gene. This model is consistent with the finding of several highly similar PPR motif–containing genes at the nuclear loci in petunia, *Brassica*, and rice that have been found to affect the expression of CMS-associated *orf*s (Bentolila et al., 2002; Desloire et al., 2003; Kazama and Toriyama, 2003). It also is consistent with the finding that alleles of restorers in *Brassica* affect the expression of genuine mitochondrial genes and that the presence of the maize *Rf3* locus affects transcripts of a chimeric region as well as known mitochondrial genes (Brown, 1999; Wen and Chase, 1999b).

A toxic sequence may rely on regulatory sequences also found next to essential mitochondrial genes. Recognition of these regulatory sequences may depend on a nucleus-encoded, mitochondrially targeted protein. If the toxic sequence invaded the population only recently, duplication and divergence of the nuclear gene may not have occurred to separate the regulation of essential genes from the toxic gene. If so, the pollen-disrupting *orf* and several normal mitochondrial genes may be dependent on the proper function of the same nuclear gene that encodes a mitochondrial gene regulatory protein. A loss-of-function mutation in such a gene could result in gametophytic fertility restoration but likely would be lethal when homozygous as a result of the disrupted expression of important mitochondrial genes in vegetative tissue, analogous to the severe defects seen in maize nonchromosomal stripe mutants (Newton, 1993), which cannot express particular essential mitochondrial genes. This scenario is consistent with the genetics of restoration in the CMS-S system. Restorer-of-fertility mutations have been identified that downregulate the CMS-associated locus in CMS-S pollen, resulting in gametophytic male fertility. Many of these mutations are homozygous lethal, presumably because they also are required for the expression of essential mitochondrial genes (Gabay-Laughnan et al., 1995). Recently, a homozygous lethal CMS-S restorer allele was shown to reduce the transcript abundance of both the CMS-associated locus and *atpA*; therefore, its wild-type allele may correspond to a nuclear gene required for *atpA* expression. Thus, screening for spontaneous restorer genes in gametophytic systems may provide a source of lines carrying mutations in nuclear genes that are required for mitochondrial gene expression (Wen et al., 2003). As more nuclear loci that affect the expression of CMS-encoding regions are cloned and sequenced, we will learn how evolution has allowed plants to flourish despite a nondiscriminating mitochondrial genome that often incorporates immigrant DNAs. Based on the wealth of information accumulated in many laboratories, we can expect that restorer loci will fall into three classes. Perhaps the largest will be composed of genes that regulate the expression of detrimental mitochondrial genome rearrangements. A second group will consist of genes that affect the abundance of particular subgenomic molecules. The third group will encompass genes that ameliorate metabolic defects caused by the expression of toxic sequences or the impaired expression of essential mitochondrial genes.

ACKNOWLEDGMENTS

We thank R. Wise, P. Simon, G. Brown, and K. Glimelius for photographs; C. Chase, C. Leaver, G. Brown, A. Alfonso, and M. Caboche for preprints; C. Hegeman-Crim and B. Eaglesham for assistance with computer graphics and photography; and S. Mackenzie for critically reviewing the manuscript. The authors' work on CMS/restorer systems has been supported by the U.S. Department of Agriculture National Research Initiative Plant Genome Program and the Rockefeller Foundation.

Received August 1, 2003; accepted January 1, 2004.

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NOTE ADDED IN PROOF

The rice PPR8-1 gene discussed in Figure 3 has recently been shown to confer fertility to rice CMS lines by transformation analysis:

Komori, T., Ohta, S., Murai, N., Takakura, Y., Kuraya, Y., Suzuki, S., Hiei, Y., Imaseki, H., and Nitta, N. (2004). Map-based cloning of a fertility restorer gene, *Rf-1*, in rice (*Oryza sativa L.*). Plant J. **37,** 315–325.